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Response surface optimization of a glycolipid biosurfactant produced by a sponge associated marine bacterium *Planococcus* sp. MMD26

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ABSTRACT

Marine sponge associated bacterium *Planococcus* sp. MMD26 was isolated and selected for biosurfactant production. Criteria for screening of biosurfactant production by MMD26 were hemolytic activity, oil displacement test and drop collapsing test. Based on the response surface methodology (RSM)-based optimization experiments the biosurfactant production was enhanced to one fold over the wild strain. The biosurfactant produced by the strain *Planococcus* sp. MMD26 was characterized as a glycolipid biosurfactant based on FT-IR, GC-MS and ^{1}H NMR spectra. The biosurfactant producing strain *Planococcus* sp. MMD26 had the ability to degrade 80% of different hydrocarbons present in the crude oil. The production of the glycolipid biosurfactant from *Planococcus* sp. MMD26 under submerged fermentation condtions revealed that the strain could be used for the pilot scale production of biosurfactant for environmental applications.

1. Introduction

Marine sponges have gained significant attention in various scientific fields with respect to the discovery of potential bioactive molecules having wide range of applications. Because of the application of these bioactive compounds in pharmaceutical and other industries, sponges are considered as a goldmine to scientists. Microbiologists have been fascinated by these unique animals when it was revealed that sponges contain abundance of an unexplored microbial diversity with promising applications in bioremediation and drug discovery (Thakur and Muller, 2004). Webster and Hill (2001) suggested several possibilities for the sponge-microorganism symbiosis: host selectively absorbs the specific symbionts; the specific symbionts grow more rapidly than any other specific microorganism or the host acquires the specific symbionts via vertical transmission from the parent sponge to the larvae. Sponges seem to acquire the symbiotic microorganisms via the feeding route (Duglas, 1994). Biosurfactants are secondary metabolites synthesized by microbes having a heterogeneous nature and surface active properties (Desai and Banat, 1997). Bacterial production of biosurfactant was first demonstrated by Bushnell and Haas (1941). These biosurfactants are produced extracellularly or on the living surfaces and exhibits an amphiphilic nature contributed by the hydrophobic and hydrophilic moieties present in the molecule which will provide the ability to decrease the surface tension and interfacial tension at the interface and surface between the fluid layers (Maier, 2003; Karanth et al., 1999). Biosurfactants are grouped into phospholipids, glycolipids, polymeric biosurfactants, lipopeptides or lipoproteins, lipopolysacharides and mycolic acids based on the chemical nature (Desai and Banat, 1997).

The increased attention towards the biosurfactants than the chemical surfactants is due to their high biodegradability, low toxicity, and effectiveness at intense temperature, pH, salinity and widespread applicability (Desai and Banat, 1997; Kosaric, 1992). Recently, diverse functions have been demonstrated for biosurfactants including emulsification activity, antiviral activity and antimycoplasmic activity (Dehghan-Noude et al., 2005). Antimicrobial action of biosurfactants against bacteria, fungi, algae and viruses is previously studied. Sorphorolipids and its structural analogues derived from *Candida bombicola* have been reported for their cytotoxic activity, anti-HIV potential and spermicidal effect (Shah et al., 2005). A potent glycolipid biosurfactant with antibiofilm activity against *Vibrio alginolyticus* was previously isolated from marine sponge associated *Nocardiopsis*MSA13A (Kiran et al., 2014) and *Brevibacterium casei* (Kiran et al., 2010). A lipopeptide biosurfactant extracted from marine *Nesterenkonia* sp. showed good antioxidant activity and protective effect against multidrug resistant

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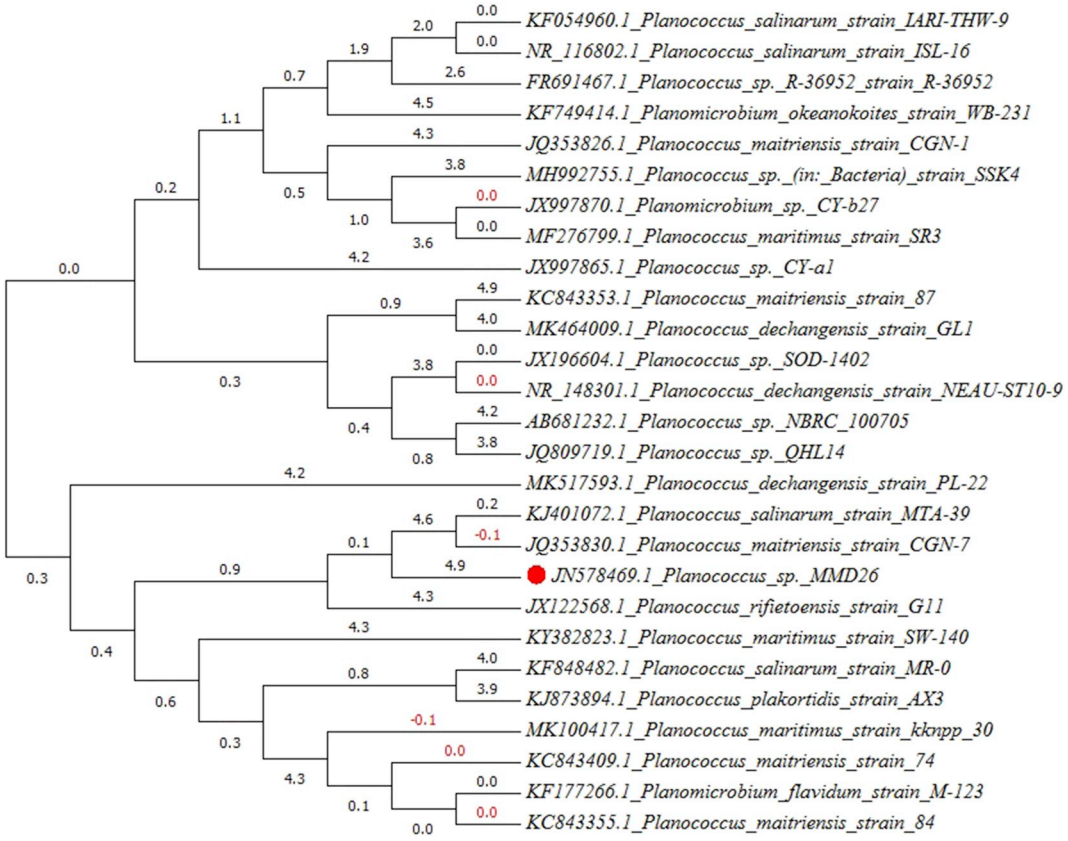

Fig. 1. The evolutionary history was inferred using the UPGMA method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) is shown next to the branches. The evolutionary distances were computed using the number of differences method and are in the units of the number of base differences per sequence. Evolutionary analyses were conducted in MEGA5.

Staphylococcus aureus (Kiran et al., 2017). Crude oil pollution in the marine environment is mainly due to standing tankers and is considered as a severe environmental problem in the current scenario (Olivera et al., 2003). It is necessary to degrade these pollutants to maintain a healthy living system from the environmental point of view. Even though hydrocarbon degradation was mainly achieved by chemical surfactants from past years, their use is limited due to their low decomposability, elevated toxicity and non-eco-friendly nature. Marine microbial community exhibits the potential to degrade these pollutants. But only a few biosurfactant producing marine microbes are explored so far, an ornithine lipid derived from *Myrides* sp. and a glucose lipid produced by *Alcaligenes* sp. (Maneerat et al., 2005; Poremba et al., 1991). Our research group established sponge associated bacteria an indicator model for monitoring heavy metal pollution in the marine environment (Selvin et al., 2009). This study anticipates to explore sponge associated marine microbes for the extraction and characterization of biosurfactants. In the present work, a hydrocarbon degrading sponge-associated marine bacterium *Planococcus* sp. MMD26 was isolated from a marine sponge *Dendrilla nigra*. The surface active compound was chemically characterized, optimized the production and hydrocarbon (crude oil) degradation potential was demonstrated.

2. Materials and methods

2.1. Sample collection and isolation of sponge associated bacteria

Marine sponges from a depth of 10–15 m were collected by SCUBA diving at Vizhinjam coast, southwest coast of India (Latitude: 8° 22′ 0.01″ N Longitude: 76° 59′ 48.01″ E). To avoid cross contamination, unbroken sponge samples were selected and used for microbial isolation. Collected sponges were packed in sterile polystyrene bags and aseptically transferred to laboratory. Sponge samples were surface sterilized by a rapid wash of 95% ethanol, sterile seawater and blotted. Sponge inner tissue of 1 cm^2 area was excised, macerated and the homogenate was serially diluted with phosphate buffered saline (PBS). All dilutions were plated in triplicates on nutrient agar (HiMedia) with 2% NaCl and Zobell marine agar (HiMedia) plates and incubated at 26 °C for 24 h to 14 days. The colonies were repeatedly streaked to obtain pure cultures and stored in nutrient agar slants (with 2% NaCl) at 4 °C.

2.2. Screening for biosurfactant production

The isolates were screened for hemolytic activity (Carillo et al., 1996), drop collapsing test (Youssef et al., 2004), oil displacement test (Morikawa et al., 1993) and emulsification activity Paraszkiewicz et al. (1992). Screening assays were performed in triplicates with distilled water as the control. The marine broth without bacterial growth was maintained as negative control.

2.3. Molecular characterization of a potential producer

The genomic DNA of bacterial endosymbiont was isolated by CTAB/NaCl method. Universal 16 S rDNA eubacterial primer (5′-GAGTTTGATCCTGGCTCAG-3′; 5′-AGAAAGGAGGTGATCCAGCC-3′) was used for the amplification of DNA. The 16S rDNA gene sequence obtained from the producer MMD26 was compared with other bacterial sequences using NCBI BLASTn (Altschul et al., 1990; Altschul et al., 1997) for their pair wise identities. Multiple alignments of these sequences were carried out by Clustal W 1.83 version of EBI (www.ebi.ac.uk/cgi-bin/clustalw/) with 0.5 transition weight. The sequence obtained was deposited in GenBank for accession number.

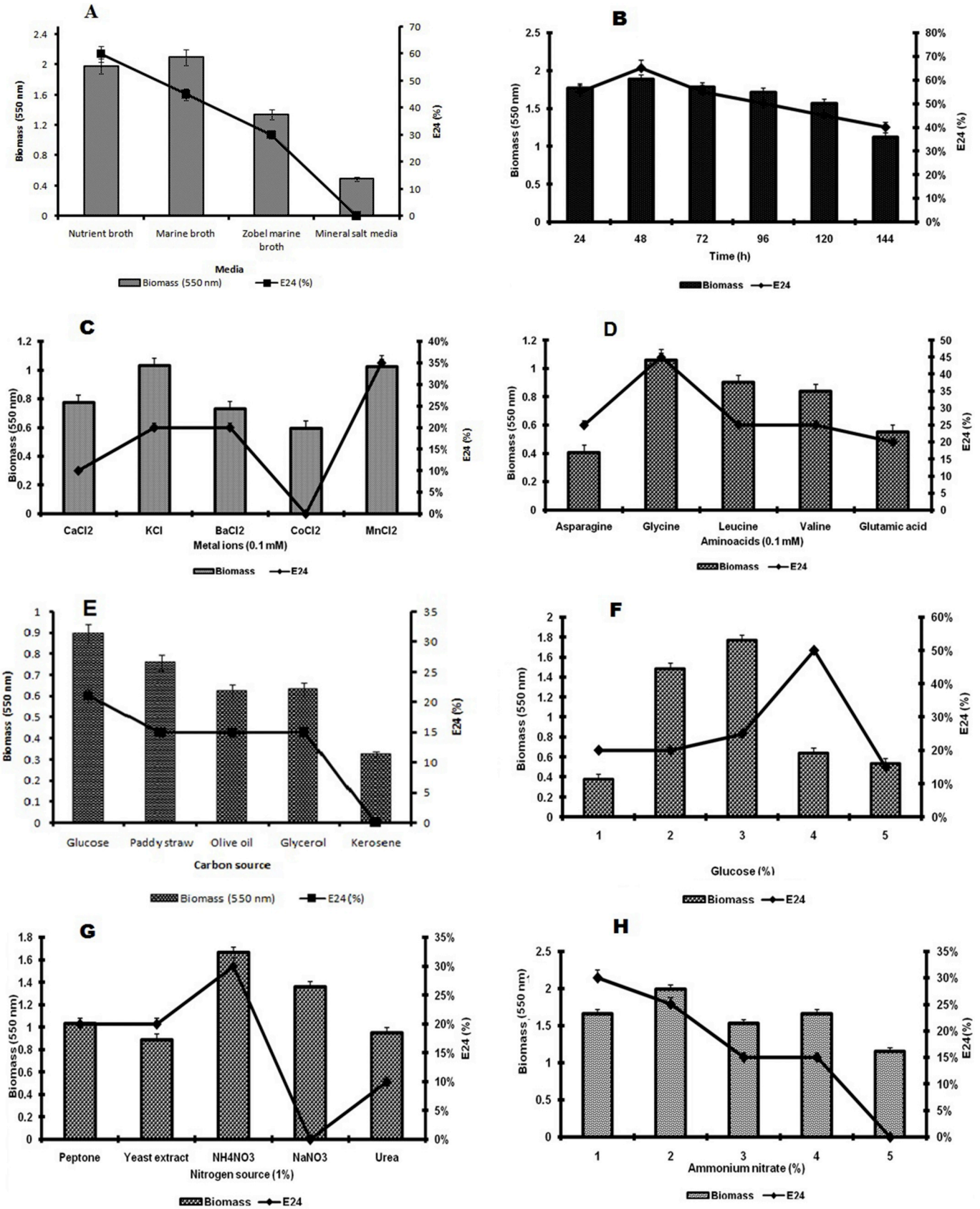

Fig. 2. Optimization of culture media for maximum emulsification activity and growth A) Fermentation media, B) Incubation time, C) Metal ions, D) Amino acids, E) Carbon source, F) Glucose concentration, G) Nitrogen source, H) Ammonium nitrate concentration.

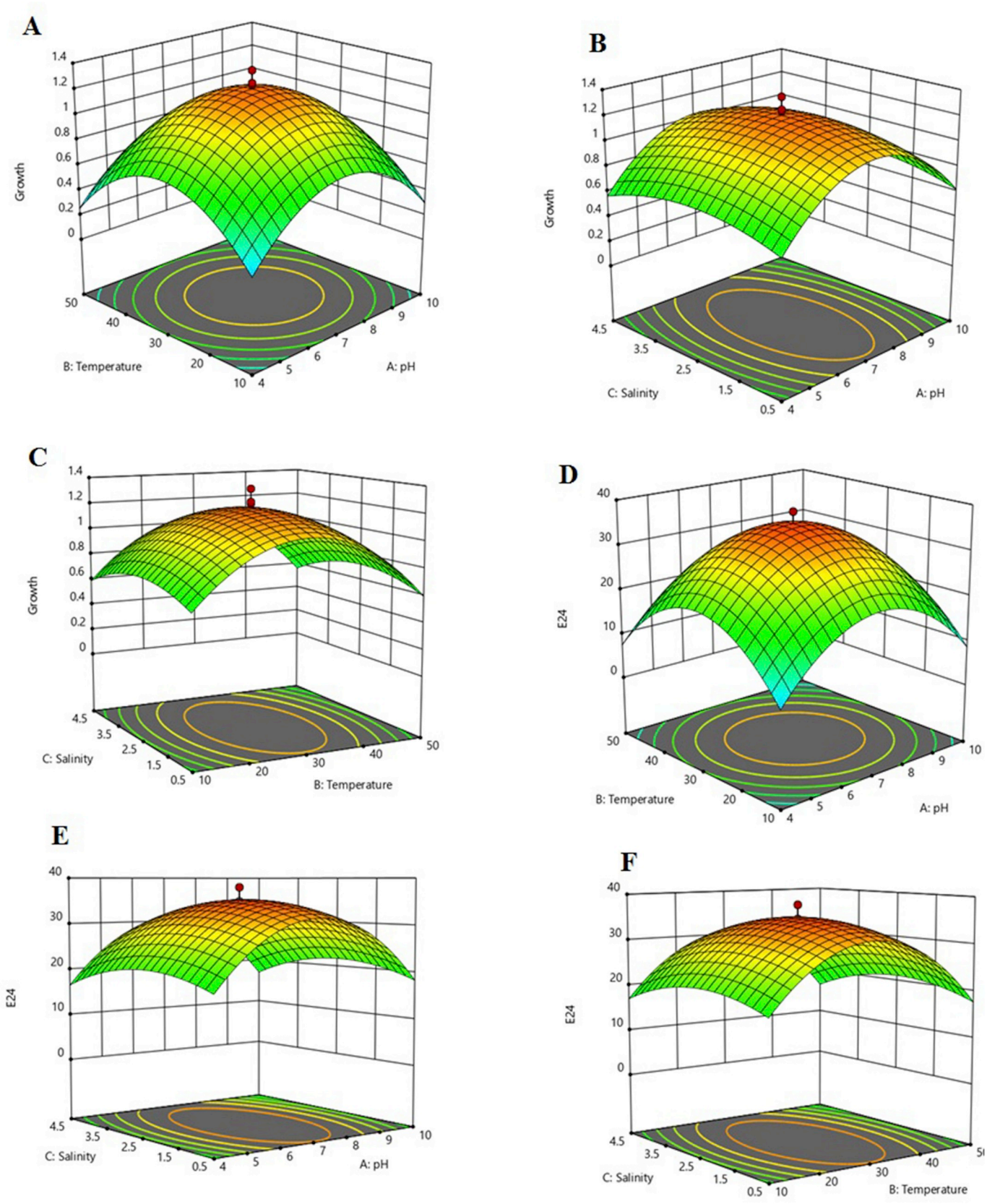

Fig. 3. Response surface plots generated using the data for maximum emulsification activity and growth. 20 Experimental runs carried out under the conditions established by CCRD (A) pH and temperature for maximum growth (B) pH and Salinity for maximum growth (C) Temperature and salinity for maximum growth (D) pH and temperature for maximum E24 (E) pH and salinity for maximum E24 (F) Temperature and salinity for maximum E24.

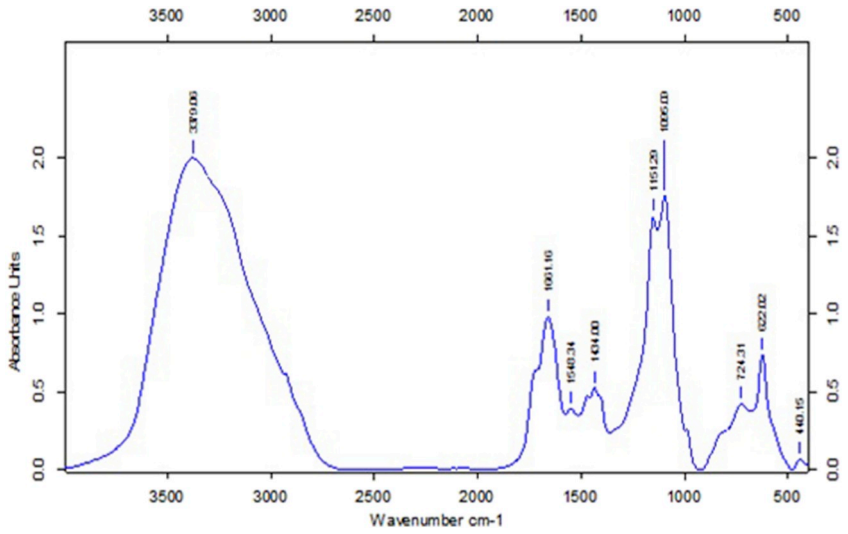

Fig. 4. FT-IR spectrum of biosurfactant from *Planococcus* sp. MMD26 showing the absorption peaks of glycolipid type biosurfactant.

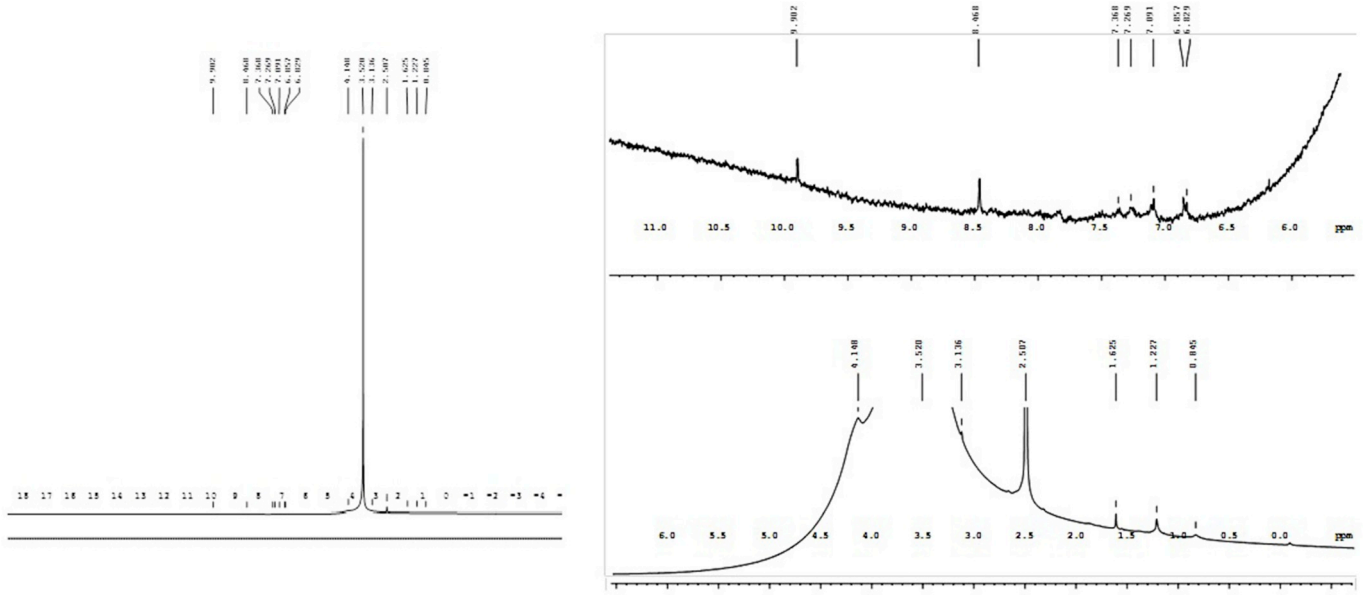

Fig. 5. ^{1}H NMR analysis of biosurfactant compound from *Planococcus* sp. MMD26.

2.4. Optimization of biosurfactant production

A loopful of overnight grown culture of MMD26 was transferred to Erlenmeyer flask containing 100 mL of nutrient broth. The flask was then incubated at 250 rpm and 30 °C for 28 h. The impact of various physical parameters and media components on biosurfactant synthesis were optimized. In all instances, parameters such as biomass and biosurfactant activity were determined. The physical parameters studied includes effect of pH (4–9), incubation temperature (10–50 °C), NaCl (0.5–3.5%) and incubation period (24–144 h). The impact of various nitrogen sources, minerals, carbon sources and amino acids were included in the optimization process. The impact of various carbon sources on biosurfactant synthesis by the MMD26 was optimized using vegetable oils (olive oil), carbohydrate (D-glucose, glycerol and paddy straw) and hydrocarbon (kerosene). The nitrogen sources optimized were categorized into inorganic sources (sodium nitrate and ammonium nitrate) and organic (urea, yeast extract and peptone). Amino acids such as (leucine, valine, glutamic acid, glycine and aspartic acid) were added to the culture medium at the concentration of 0.1 M. The result of the incorporation of various metal ions on biosurfactant production was evaluated by supplementing them to the nutrient broth (Himedia) at a concentration of 0.1 mM.

2.5. Optimization of production media using experimental designs

Four variables that have effect on biosurfactant production were identified by one factor at a time experiments. To find the most significant factor a 3-level fractional factorial design was identified (Montgomery, 1991). According to the 3-level five variable concept, a comprehensive matrix was created. Each independent variable was investigated at a high (+1) and a low (−1) level. Runs of center points were included in the matrix and the runs are randomized for statistical reasons. The variables that showed maximum biosurfactant production were identified on the basis of confidence levels above 95 percent ($P < 0.05$). All analysis was carried out using Design Expert, Ver. 8.0.4 (Stat-Ease Inc., Minneapolis, USA). The insignificant variables are maintained as constant values ('0' coded level) as in the 3-level fractional factorial design.

2.6. Culture condition and purification of biosurfactant

The bacterial strain MMD26 was cultured in 1 L nutrient broth with 2% NaCl (HiMedia) and grown for 48 h at 30 °C. The extracellular production of biosurfactant was determined at different time intervals and the samples were tested for emulsification activity as described above. Culture supernatant was obtained by centrifugation and then acidified with 0.1 N HCl to obtain the pH 2.0. Extraction was performed with the addition of equal volume of solvents including chloroform, ethyl acetate, methanol, diethyl ether and dichloromethane. Solvent extract with high emulsification activity was selected for further studies and for the determination of the chemical characterization of biosurfactant compound.

2.7. Chemical characterization of biosurfactant compound

To purify the biosurfactant compound, extract was subjected to silica gel column chromatography (60–120 mesh) eluted with methanol at a flow rate of 0.5 ml/min. The active fraction containing biosurfactant was lyophilized (Yamato DC 400) and was subjected to FT-IR analysis. IR spectra were recorded on a Bruker IFS113v FTIR spectrometer, in the 4000–400 cm^{-1} spectral region. An Agilent GC system (SHIMADZU – GC – 2014 Model) equipped with a fused silica capillary tube was used to analyze the components in this active fraction. For the structure prediction one dimensional ^{1}H NMR spectra were recorded on a 300 MHz NMR spectrometer (Bruker, Germany).

2.8. Biodegradation of hydrocarbon in crude oil

2.8.1. Screening test

The activity of crude oil degradation was screened on Luria Bertani (LB) agar plate containing crude oil according to the method of Kim et al. (1997). The bacterial adhesion to the hydrocarbons (BATH) assay was performed to check the hydrophobicity of the isolate MMD26 (Rosenberg and Rosenberg, 1985). Hydrophobicity is expressed as the percentage of adherence to crude oil, according to the calculation 100 × (1- OD of the aqueous phase/OD of the cell suspension).

3. Results

3.1. Isolation and screening of biosurfactant producer

Marine bacteria was isolated from a marin sponge *Dendrilla nigra*, altogether 55 bacterial strains were isolated. Among them only 10 isolates showed positive results and the strain MMD26 was considered as the potential biosurfactant producer based on the stable emulsification activity. The scope of the present study emphasis on the production, optimization, chemical characterization and degradation of hydrocarbon by a marine bacterium MMD26. Results for screening of biosurfactant production by *Planococcus* sp. MMD26 showed hemolytic activity with a clear zone diameter of 7 mm around the colony. In drop collapse test, a flat drop was observed whereas in oil displacement method, a clear diameter of 8 mm corresponding the area of 50.24 mm^2. The strain MMD26 showed an emulsification activity of 45%. Based on these observations, it was confirmed that the strain MMD26 was a potent biosurfactant producing marine bacterium.

3.2. Molecular characterization of biosurfactant producer

From the classifier programme of RDPII, the taxonomic relationship of 16 S rDNA sequences of the isolate MMD26 was recovered. Megablast tool of GenBank was used to blast the 16 S rDNA sequence of MMD26 (http://www.ncbi.nlm.nih.gov/). Based on the sequence analysis and the phylogenetic tree constructed showed the isolate MMD26 belongs to *Planococcus* sp. (Fig. 1). The sequence was deposited in GenBank, NCBI with an accession number JN578469.

3.3. Optimization of biosurfactant production

Environmental features and growth circumstances such as oxygen availability, temperature and agitation also influence biosurfactant production. The results of time course for biosurfactant synthesis showed the production was growth associated. Optimum growth and emulsification activity was observed at 48 h of incubation and emulsification activity decreased with increase in incubation time and least emulsification activity was observed at 144 h. Maximum emulsification activity of 50% was obtained by using glucose as sole carbon source (4%) and ammonium nitrate as nitrogen source (1%) (Fig. 2a). The isolate MMD26 utilized glycine and manganese chloride (metal) when compared to other amino acids and metal ions. Based on the results obtained from the optimization process, nutrient broth showed a drastic growth and biosurfactant production when compared to other growth media used in this study (Fig. 2b).

3.4. Optimization of biosurfactant production by experimental design for MMD26

Response surface methods were used to build a model for modeling growth, emulsification index and process variables. Response surface plots and contour plots for maximum growth and emulsification index are given in Fig. 3.The following quadratic polynomial equation showed best fit of the data.

$$Y_1 = -2.60874 + 0.731818\,A + 0.069811\,B + 0.171688\,C - 0.000110\,A*B - 0.000479\,A*C + 0.000041\,B*C - 0.051733\,A^2 - 0.001164\,B^2 - 0.035788\,C^2$$

Where Y_1 is the growth (OD at 550 nm), A, B, and C are coded values for pH, temperature (°C) and salinity (%). The quadratic equation showed best fits of the data for maximum emulsification index. Fig. 3A showed the effect of pH and temperature on growth of the biosurfactant production where maximum growth was observed with pH 7 and at a temperature of 30 °C (Fig. 3A and B).

The following quadratic polynomial equation showed best fit of the data for maximum emulsification activity.

$$Y_{2=} - 76.92697 + 21.67260\,A + 2.08430\,B + 4.51905\,C - 0.005833A*B + 0.016667\,A*C + 0.003750\,B*C - 1.53364\,A^2 - 0.034507\,B^2 - 1.02000\,C^2$$

Where Y_2 is the emulsification index (E24%) and A, B, and C are coded values for pH, temperature (°C) and salinity (%). Maximum emulsification activity was observed with 2.5% salt concentration in the optimized production media (Fig. 3D).

3.5. Characterization of biosurfactant compound

Chemical characterization of the biosurfactant MMD26 by FT-IR analysis showed a broad peak at 3387 cm^{-1} corresponds to OH group, absorption at 1658.21 cm^{-1}corresponds to C═C and 1544.58 cm^{-1} corresponds to C—C absorption at 1464 cm^{-1} corresponds to scissoring CH_2, absorption at 1150 cm^{-1} corresponds to C—O—C strecthing indicates the presence of carbohydrate group and absorption at 623 cm^{-1} may be due to C—H deformation (Fig. 4). The mass spectrum of the purified compound was measured in GC-MS giving signals at a retention time of 28.37 min. The^{1}H NMR analysis of biosurfactant compound from *Planococcus* sp. MMD26 gave signals at 7.474–7.270 which corresponds to -CH_2 = CH- groups, 0.84 ppm showed the presence of -CH_3), peak at 1.61 and 1.2 indicates methyl (CH_3) groups (and peaks at 3.5 and 3.2 ppm may be due to the protons of sugar, 2.49 -ppm may be due to the presence of -$COCH_3$ (Fig. 5).

3.6. Hydrocarbon degradation

3.6.1. Screening test

In plate assay *Planococcus* sp. MMD26 exhibited a marked crude oil degrading activity as visualized by the clear zone that developed around the colony after 24 h of incubation at 37 °C. In BATH assay the strain *Planococcus* sp. MMD 26 had higher cell hydrophobicity (84%) correlated to the hydrocarbons in crude oil. In this case the organism *Planococcus* sp. MMD 26 showed relatively low affinity towards the hydrocarbons in early exponential phase. However, the affinity increased as the culture reached the early stationary phase.

4. Discussion

In the present study a sponge associated marine bacterium *Planococcus* sp. MMD26 was isolated from amarine sponge *D. nigra*. Literature showed the presence of *Planococcus* sp. in marine sponge microbiome (Kaur et al., 2012). The environmental factors and chemical constitution of the culture medium impacts cell augmentation and biosurfactant synthesis (Guerra-Santos et al., 1986). A better understanding of the environmental factors and media components and their ideal regulation can, thus, be utilized to enhance the biosurfactant synthesis. The pH of the culture medium is one among the prime factors that can influence surfactant synthesis and bacterial proliferation. In this study cell growth and biosurfactant production was optimum at pH 7 with an emulsification activity of 45%. The habitat temperature was analogous to the incubation temperature mostly used for surfactant production (Kim et al., 1997). The sources of carbon used commonly in biosurfactant production can be split into three categories: hydrocarbons, carbohydrates and vegetable oils. In this study, the carbon sources assessed were carbohydrate sources (paddy straw, glycerol and D-glucose), vegetable oils (olive oil), and hydrocarbon (kerosene). The isolate MMD 26 utilized glucose as the carbon source with the emulsification activity of 50%. Nitrogen source enacts a vital role in the synthesis of surface-active compounds by microorganisms (Nitschke et al., 2005). Biosurfactant synthesis was highly influenced by the source of nitrogen as the synthesis of protein and enzyme required for microbial metabolism confide in nitrogen source (Vandana and Singh, 2018). The nitrogen sources assessed were inorganic sodium nitrate and ammonium nitrate and organic yeast extract, peptone and urea. The concentration of L-amino acid in the medium affected the surfactant structure to either Val-7 or Leu-7 surfactant (Guerra-Santos et al., 1984). Likewise, the supplementation of L-glutamic acid and L-asparagine increased the production of lichenysin-A by two and four folds respectively in *B. licheniformis* BAS50 (Yakimov et al., 1998). Wei and Chu (1998) reported the stimulatory effect of iron (II) and iron (III) on cell growth and surfactant synthesis by a *Bacillus* species. Pollution of the sea and coastlines is particularly problematic in both its scale and severity of its ecological perturbation (Yakimov et al., 1998). Marine microbes offer a new avenue for the synthesis of surface-active compounds (Tripathi et al., 2018). Karlapudi et al. (2018) reported *Pseudomonas aeruginosa,* from oil polluted sea water has the potential to degrade hydrocarbons like octadecane, hexadecane etc. Strain *Planococcus* MAE2 isolated from crude-oil-contaminated intertidal beach sediments degrade a wide range of branched and normal alkanes up to C33 (Engelhardt et al., 2001). This study provides a novel insight on hydrocarbon-degradation by *Planococcus* sp. MMD26. BATH assay was carried out to test the hydrophobicity of bacterial cell surface. The cells grown in hydrocarbon – containing media had higher values of hydrophobicity than cells grown in water-soluble media. This signifies that the biosurfactant not only aids in emulsification but also enacts in cell surface hydrophobicity change to enhance the microbial cell affinity for the substrate to enable their bioavailability (Rosenberg and Rosenberg, 1985).

5. Conclusion

This report showed the glycolipid biosurfactant production and its optimization using a marine *Planococcus* sp. MMD26. Thus, such organism may play a vital role in the biodegradation of hydrocarbon contaminants in the environment and have potential use in boosting bioremediation processes. Due to the hydrocarbon degradation potential of *Planococcus* sp. MMD26, the biosurfactant identified in this study can be a potential resource for environmental cleaning process.

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